Sulforhodamine B colorimetric assay for cytotoxicity screening

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The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods. The method not only allows a large number of samples to be tested within a few days, but also requires only simple equipment and inexpensive reagents. The SRB assay is therefore an efficient and highly cost-effective method for screening.

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INTRODUCTION

The sulforhodamine B (SRB) assay, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxicity screening[1]. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions[2]. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.

The strong intensity of SRB staining allows the assay to be carried out in a 96-well format. Skehan *et al.*[1] showed that the assay can detect densities as low as 1,000–2,000 cells per well, and with a signal-to-noise ratio of 4.83 at a density of 5,000 cells per well. This level of sensitivity is comparable to those of fluorescent dye-staining methods, and is superior to those of other protein-staining methods using conventional visible dyes[1,3]. Results from the SRB assay exhibit a linear dynamic range over densities of 7,500–180,000 cells per well, corresponding to ~1–200% confluence[1]. Furthermore, the SRB method has proven to be practical, because after the TCA-fixed and SRB-stained cell monolayers are dried they can be stored indefinitely. Color extracted from SRB-stained cells is also stable. With its high level of sensitivity, adaptability to the 96-well format and endpoint stability, the SRB assay is well suited to large-scale screening applications, as well as research. This assay has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines[4]. Other cell-growth assays have been employed to assess drug efficacy against both intracellular pathogens and host cells simultaneously in co-cultures[5,6], and it is possible to use the SRB method for this purpose. An antiviral assay using SRB has been developed at our facility for the screening of natural compounds against herpes simplex virus type 1 (HSV-1)[7]. In addition, the SRB method has also been shown to be effective for *in vitro* testing of cancer cell sensitivity to radiation, and for the study of interactions between radiotherapy and chemotherapy, with a sensitivity comparable to that of the standard clonogenic assay[8,9].

The effectiveness of the SRB assay is frequently compared to that of another method using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay requires cellular metabolic activity to convert the colorless tetrazolium to the purple-colored formazan dye[10]; therefore, it detects only viable cells, whereas the SRB method does not distinguish between viable and dead cells. This difference, however, does not compromise the ability of the SRB assay to detect cytotoxic effects of a drug. Studies undertaken by several groups showed that results from the SRB assay correlated well with those of the MTT assay, although the IC_{50} values of compounds tested using the SRB method were slightly higher[11–13]. However, the SRB assay has several advantages over the MTT assay. For example, some compounds can directly interfere with MTT reduction without having any effects on cell viability[10], while SRB staining is rarely affected by this type of interference. Furthermore, SRB staining is independent of cell metabolic activity; therefore, fewer steps are required to optimize assay conditions for specific cell lines than in the MTT assay[14].

The application of the SRB assay is limited to manual or semiautomatic screening due to the multiple washing and drying steps, which, at present, are not amenable to automation. This method nevertheless provides an efficient and sensitive tool for screening, especially for use in less well-equipped laboratories. The protocol presented here has been slightly modified from the original SRB assay described by Skehan *et al.*[1], because we found that the concentrations of TCA and SRB required for the fixation and staining of several cell lines could be decreased, therefore reducing the health risks to technicians and the burden of toxic-waste disposal.

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MATERIALS

REAGENTS

- Adherent cell line of interest
- Appropriate culture medium
- $NaHCO_3$ (Sigma, cat. no. S5761)
- 10 mM minimal essential medium (MEM) non-essential amino-acid solution (Invitrogen, cat. no. 11140)
- MEM (Eagle) supplemented with 2 mM L-glutamine and Earle's balanced salt solution (MEM/EBSS; Hyclone, cat. no. SH30008)
- 100 mM sodium pyruvate (Hyclone, cat. no. SH30239)
- FBS (PAA Laboratories, cat. no. A11-043)
- 10 mg ml^{-1} bovine insulin in 25 mM HEPES, pH 8.2 (Sigma, cat. no. I0516)
- 2.5% (wt/vol) trypsin solution (Invitrogen, cat. no. 15090)
- 0.5% (wt/vol) phenol red solution (Sigma, cat. no. P0290)
- 0.48 mM versene-EDTA
- 0.4% (wt/vol) trypan blue in 0.81% (wt/vol) NaCl and 0.61% (wt/vol) KH_2PO_4 (Sigma, cat. no. T8154)
- Dimethyl sulfoxide (DMSO; Sigma, cat. no. D4540)
- Positive control: doxorubicin (Sigma, cat. no. D1515) or ellipticine (Sigma, cat. no. E3380)
- 10% (wt/vol) TCA
- 1% (vol/vol) acetic acid
- 0.057% (wt/vol) SRB (Fluka, cat. no. 86183) in 1% (vol/vol) acetic acid
- 10 mM unbuffered Tris base solution

EQUIPMENT

- 96-well clear flat-bottom polystyrene tissue-culture plates (Corning, cat. no. 3599)
- 75-ml tissue-culture flasks or 100 mm tissue-culture plates (Corning, cat. no. 430725)
- Inverted microscope (TMS, Nikon)
- Multiwell microplate reader (Wallac Victor V, PerkinElmer)
- Multichannel pipette (Gilson)
- Gyratory plate shaker (Model 4625, Lab-Line)
- Statistical analysis software or IC_{50}-calculation software (SigmaPlot, SPSS or MicroWin, Mikrotek)

REAGENT SETUP

Test samples These are crude extracts of naturally occurring or pure compounds. Depending on the extraction method and the compound solubility, samples can be dissolved in DMSO, deionized water or ethanol, and subsequently diluted in culture media. Most samples can be dissolved and stored in DMSO; therefore, we generally prepare samples in 100% DMSO, and use final concentrations of the sample and the DMSO of 50 μg ml^{-1} and 0.5% (vol/vol), respectively, in primary screening. However, difficult-to-dissolve samples might be tested at lower concentrations and, if higher or lower assay stringency is desired, sample concentration can be varied accordingly, provided that the final concentration of solvent does not become toxic.

Cell-growth medium Use the medium recommended for each cell line. For the cell lines mentioned in this protocol, use MEM/EBSS adjusted to 1.5 g ml^{-1} $NaHCO_3$, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 10% (vol/vol) FBS. For MCF-7, growth medium must be supplemented with 0.01 mg ml^{-1} bovine insulin.

Cells for plating 2 or 3 d before performing the assay, grow cells in 75 ml tissue-culture flasks or 100 mm tissue-culture dishes containing growth medium. Cells that are ready for plating should be healthy looking, but should not become too dense.

PBS Dissolve 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl and 0.2 g KH_2PO_4 in 800 ml deionized water, adjust pH to 7.4 with HCl, bring the volume to 1 l with deionized water and sterilize by autoclaving.

0.48 mM versene-EDTA Dissolve 0.2 g EDTA and 1.5 ml of 0.5% (wt/vol) phenol red solution in PBS, and sterilize by autoclaving.

0.25% (wt/vol) trypsin in versene-EDTA Dilute 2.5% (wt/vol) trypsin solution 10 times with 0.48 mM versene-EDTA.

10% (wt/vol) TCA ! CAUTION This chemical is highly corrosive and handling should be carried out with proper protection in a chemical fume hood. Pipetting appliances should be made of corrosion-resistant materials.

PROCEDURE

Compound preparation

1| Dissolve dried sample with 100% (vol/vol) DMSO to 10 mg ml^{-1}.

2| For primary screening, dilute the dissolved compound to 1 mg ml^{-1} with sterile deionized water. For IC_{50} determination, make a twofold serial dilution from 1 mg ml^{-1} to 15.625 μg ml^{-1} in 10% (vol/vol) DMSO. Mix compound solution by pipetting thoroughly several times after each transfer.

▲ CRITICAL STEP To prevent attachment of the compound to the plasticware, always use polypropylene microcentrifuge tubes or 96-well plates for compound dilution. Pipette tips should be changed after each transfer.

3| Prepare six concentrations of positive controls from 160 to 5 μg ml^{-1} using twofold serial dilution in 10% (vol/vol) DMSO.

4| Add 10 μl test sample in 10% (vol/vol) DMSO to each compound well of a 96-well tissue-culture plate in triplicate. Add 10 μl of 10% (vol/vol) DMSO into each negative-control well. Add 10 μl ellipticine (or doxorubicin) in 10% (vol/vol) DMSO into each positive-control well.

Cell preparation

5| Prior to performing the assay, remove medium from cell monolayers and wash the cells once with sterilized PBS.

6| Remove PBS and add just enough 0.25% (wt/vol) trypsin in versene-EDTA to evenly cover the cell-growth surface. When the cells start to dissociate, use a sterilized plastic or glass pipette to disperse them from the culture surface with 10 volumes of culture medium containing FBS, and mix to obtain a homogeneous cell suspension.

7| Transfer the cell suspension to a sterilized polypropylene tube. Determine the cell concentration by counting in a hematocytometer chamber under a microscope, using a 1:1 mixture of cell suspension and 0.4% (wt/vol) trypan blue solution. Do not proceed with the assay if a large portion of the cells looks unhealthy and stains with trypan blue dye.

8| Adjust the cell concentration with growth medium to obtain an appropriate cell seeding density. From our experience, a seeding density of 1.9×10^4 cells per well provides satisfactory results with epithelial and fibroblast cell lines, such as the BCA-1 and MCF-7 human breast carcinoma cell lines, the KB human squamous cell carcinoma cell line, and the African green monkey kidney cell line (Vero).

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Exposure of cells to test compound

9| Add 190 μl cell suspension to the assay plates prepared in Step 4. Occasionally mix the cell suspension during plating to ensure an even distribution of the cells. When assaying a large number of test compounds, it is practical to add cell suspension to wells already containing compounds, in order to reduce the time that the cells spend outside the incubator and to minimize the chance of contamination. In addition, adding a large volume of cell suspension to a small volume of sample allows the liquid to mix more thoroughly and thus reduces the need for additional agitation. Although it is possible that test compounds can interfere with cell attachment, the small number of hits obtained during our cancer cell line screening suggests that the chance of detecting such an effect is negligible (K.K. *et al.*, unpublished data).

▲ **CRITICAL STEP** Non-homogeneous plating density is a major cause of error in cell growth-inhibition assays.

10| Set aside a plate containing only cell suspension in three columns for a no-growth control (day 0). Incubate the plate at 37 °C in a humidified incubator with 5% CO_2 until cell attachment completes. For most cell lines, this requires ~2–3 h. Then, proceed to Step 12 to fix the cell monolayer.

11| Incubate the remaining assay plates at 37 °C in a humidified incubator with 5% CO_2 for 72 h.

Cell fixation and staining

12| Without removing the cell culture supernatant, gently add 100 μl cold 10% (wt/vol) TCA to each well, and incubate the plates at 4 °C for 1 h. Our experience with the BCA-1, KB, MCF-7 and Vero cell lines indicates that at a final TCA concentration of 3.3% (wt/vol), these cells can be fixed as effectively as at 10% (wt/vol), which is the concentration used in the original method[1]. However, fixation of loosely attached cell lines will require higher TCA concentrations and extended incubation at 4 °C. Additional steps that promote cell contact with the well bottom, such as allotting time for cells to settle or centrifugation, are likely to promote the attachment of a single-cell suspension, but do not guarantee the attachment of suspension cells growing in aggregates[1]. A trial experiment should be carried out to determine the optimal fixing conditions before performing an assay with new cell lines.

13| Wash the plates four times with slow-running tap water via plastic tubing connected directly to a faucet, and remove excess water using paper towels. Then, use a blow dryer to completely dry the plates or allow them to air-dry at room temperature (20–25 °C).

▲ **CRITICAL STEP** Do not inject the water stream directly onto the bottom of the wells, as this can cause the cell monolayer to detach.

■ **PAUSE POINT** After fixing and drying, the plates can be stored indefinitely at room temperature.

? TROUBLESHOOTING

14| Add 100 μl of 0.057% (wt/vol) SRB solution to each well. Leave at room temperature for 30 min and then quickly rinse the plates four times with 1% (vol/vol) acetic acid to remove unbound dye. Use a blow dryer to dry the plates or allow them to dry at room temperature.

▲ **CRITICAL STEP** Insufficient removal of excess dye causes overestimation of cell mass, whereas excessive washing causes the opposite result due to bleaching of protein-bound dye. Therefore, it is crucial to rinse the plates several times, and the rinses should be carried out as quickly as possible.

■ **PAUSE POINT** Stained and dried plates can be stored indefinitely at room temperature.

OD measurement and analysis of results

15| Add 200 μl of 10 mM Tris base solution (pH 10.5) to each well and shake the plate on a gyratory shaker for 5 min to solubilize the protein-bound dye. Alternatively, if a shaker is not available, SRB can be solubilized after 30 min in 10 mM Tris base solution.

16| Measure the OD at 510 nm in a microplate reader. Although the maximum absorbance of SRB can be achieved at 564 nm, it is recommended that suboptimal wavelengths of 490–530 nm should be used to avoid measurement in a nonlinear portion of the range[1]. Alternatively, the amount of SRB can be measured fluorometrically at excitation and emission wavelengths of 488 and 585 nm, respectively.

? TROUBLESHOOTING

17| Calculate the percentage of cell-growth inhibition using the formulae below. For primary screening, we use a threshold of 50% cell-growth inhibition as a cut-off for compound toxicity against cell lines. However, the threshold can be varied according to toxicity criteria defined by the investigators. For IC_{50} determination, plot a dose–response curve between the compound

concentration and percent growth inhibition. IC_{50} values can be derived using curve-fitting methods with statistical analysis software or IC_{50} calculation software.

$$\% \text{ of control cell growth} = \frac{\text{mean } OD_{sample} - \text{mean } OD_{day\,0}}{\text{mean } OD_{neg\ control} - \text{mean } OD_{day\,0}} \times 100$$

$$\% \text{ growth inhibition} = 100 - \% \text{ of control cell growth}$$

It is possible to use the SRB assay to determine the LD_{50} values of compounds[15] from the dose–response relationship between the compound concentration and the percentage of cells killed, which is calculated using the formula below.

$$\% \text{ cells killed} = 100 - \frac{\text{mean } OD_{sample}}{\text{mean } OD_{day\,0}} \times 100$$

● TIMING

Timeline (estimated for an assay run with 336 samples in 12 plates)

Steps 1–4: 2 h
Steps 5–9: 2 h
Step 10: ~2–3 h for most cell lines
Step 11: 72 h
Steps 12 and 13: 2.5 h or 1 d depending on whether the plates are blow-dried or air-dried
Step 14: 1.75 h or 1 d depending on whether the plates are blow-dried or air-dried
Steps 15 and 16: 1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Step 13: Cell detachment.	TCA concentration too low.	Set up a trial experiment to select optimal TCA concentration.
	Washing steps too harsh.	Use slow-running water.
Step 16: Error of OD values.	Cell detachment.	See Step 13.
	Incomplete removal of excess SRB.	Wash plates more thoroughly with 1% (vol/vol) acetic acid, while minimizing the time spent in washing step.
OD values too low, low assay sensitivity (percent growth inhibition of positive control too low) or OD values exceed the linearity range.	Cell-seeding density too low, cell growth reaches plateau phase or cell-seeding density too high.	Determine optimal starting cell density. Seed various numbers of cells (i.e., 1,000–20,000 cells per 190 µl medium per well) into 96-well tissue-culture plates. Follow Steps 9 and 11–16. Then determine the correlation coefficient between starting cell numbers and OD values. The cell-seeding density that produces OD in the exponential increase phase range, but not exceeding the linearity range of the absorbance reading, should be selected.

ANTICIPATED RESULTS

Generally, results obtained from the SRB assay should fall within the linearity limit (up to OD 1.5–2.0) with the $OD_{neg\ control}$ being four to six times higher than the $OD_{day\ 0}$, depending on the cell line used. If the absorbance of the sample exceeds the linearity range, an aliquot of extracted dye solution can be diluted and re-read. Typical dose–response curves of ellipticine and doxorubicin are shown in **Figure 1**.

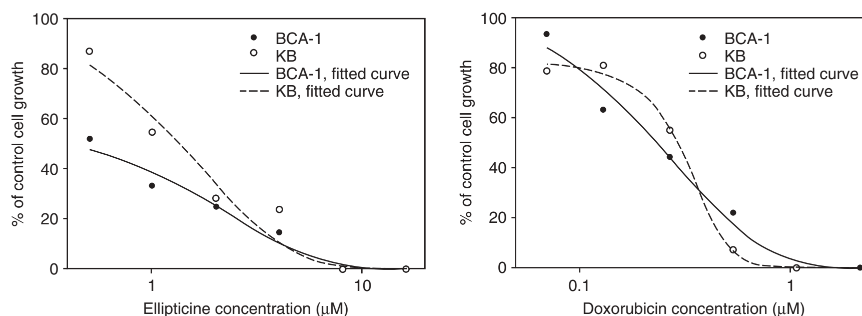

Figure 1 | Dose–response analysis of ellipticine and doxorubicin toxicity against BCA-1 and KB cell lines using the SRB assay in a 96-well format.

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